Mycobacterium tuberculosis Growth Control by Lung Macrophages and CD8 Cells from Patient Contacts

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Rationale: **Healthy household contacts (HHCs) of patients with active pulmonary tuberculosis are exposed aerogenically to *Mycobacterium tuberculosis* (*Mtb*), thus permitting the study of protective local immunity.**

Objectives: **To assess alveolar macrophage (AM) and autologous blood CD4 and CD8 T-cell–mediated *Mtb* growth control in HHCs and healthy, unexposed community control subjects (CCs).**

Methods: **AMs were infected with *Mtb* strains $H_{37}Ra$ and $H_{37}Rv$ at multiplicities of infection 0.1 and 1. *Mtb* colony-forming units were evaluated on Days 1, 4, and 7.**

Main Results: **CD8 T cells from HHCs in 1:1 cocultures with AMs significantly ($p < 0.05$) increased *Mtb* growth control by AMs. In CCs, no detectable contribution of CD8 T cells to *Mtb* growth control was observed. CD4 T cells did not increase *Mtb* growth control in HHCs or in CCs. IFN-γ, nitric oxide, and tumor necrosis factor were determined as potential mediators of *Mtb* growth control in AMs and AM/CD8 and AM/CD4 cocultures. IFN-γ production in AM/CD4 was twofold higher than that in AM/CD8 cocultures in both HHCs and CCs ($p < 0.05$). Nitric oxide production from AMs of HHCs increased on Days 4 and 7 and was undetectable in AMs from CCs. IFN-γ and nitric acid concentrations and *Mtb* growth control were not correlated. Tumor necrosis factor levels were significantly increased in AM/CD8 cocultures from HHCs compared with AM/CD8 cocultures from CCs ($p < 0.05$).**

Conclusion: **Aerogenic exposure to *Mtb* in HHCs leads to expansion of *Mtb*–specific effector CD8 T cells that limit *Mtb* growth in autologous AMs.**

Keywords: interferon type II; *Mycobacterium tuberculosis*; nitric oxide; T lymphocytes, effector; macrophages, alveolar

The human lung is the primary site of respiratory uptake of aerosolized droplet nuclei containing *Mycobacterium tuberculosis* (*Mtb*) and of tuberculosis (TB) in the nonimmunocompromised host (1–4). Hence, the study of immune responses in the lung compartment provides a window into the fundamental biological interactions at the interface of *Mtb* with the host immune cells.

Epidemiologic studies suggest presence of human protective immunity against *Mtb*. The proportions of individuals that undergo primary infection with *Mtb* or reactivation disease after aerogenic *Mtb* exposure are usually low (3, 5, 6). Healthy household contacts (HHCs) of patients constitute a protected group, as only 5 to 10% of individuals exposed in households develop reactivation TB during their lifetime (3) and 50 to 75% do not acquire *Mtb* infection as determined by tuberculin skin testing (TST) (7, 8). By inference, it can thus be assumed that the local alveolar environment of HHC during its initial interaction with *Mtb* efficiently contains or abrogates the infection in the majority of cases despite sustained aerogenic *Mtb* exposure.

After the respiratory uptake of *Mtb* in aerosolized droplet nuclei, alveolar macrophages (AMs) are believed to be among the first cells to phagocytose, contain, and kill *Mtb* and to become targets of cytotoxic T-lymphocyte and CD8 effector T-cell activity (9–12). As mediators of innate and adaptive immune responses, *Mtb*-infected AMs release cytokines and chemokines (13–19). AMs probably are also stimulated *in situ* by cytokines such as IFN-γ and tumor necrosis factor α (TNF-α), which affect the bactericidal activity and control of *Mtb* (20).

There is general consensus that CD4 T cells play a pivotal role in human protective immunity against *Mtb* (21–23). CD4 T cells are the predominant source of IFN-γ and interleukin 2 (IL-2), capable of cytotoxicity against *Mtb*-infected target cells (24), and critical for the induction of protective-memory immunity, delayed-type hypersensitivity responses, and the development and maintenance of CD8 T-cell responses during *Mtb* infection (25).

CD8 T cells play an essential role as killer cells of *Mtb*-infected targets (26, 27). CD8 T cells lyse *Mtb*-infected macrophages via a Fas-independent granule exocytosis pathway (28–30) and the Fas–FasL interaction (12), which results in the apoptotic death of *Mtb*-infected target cells. Human peripheral CD8 T cells show *Mtb* specificity and recognize *Mtb* antigens, such as ESAT-6 (31), 19kD (32), and *Mtb*39 (33), in an HLA-1 dependent fashion (11, 31, 34–37) and contribute to the production of IFN-γ (38, 39) and TNF-α on stimulation with *Mtb* (39).

IFN-γ and nitric oxide (NO) are key mediators of cell-mediated antimycobacterial activities. IFN-γ is crucial in the resistance to mycobacteria (40–43); its activity is mediated through the regulation of an array of genes that include reactive nitrogen intermediates. NO is released from *Mtb*-infected human blood monocytes (MNs) (44, 45) and AMs (46–48), confers antimycobacterial activity against *Mtb* (45, 48), and is present in human TB lung granulomas (49).

Our earlier studies in HHCs revealed increased frequencies of *Mtb* antigen 85–specific immune responses in peripheral blood mononuclear cells (PBMCs) (50) and bronchoalveolar cells (BACs) (51). These findings in BACs suggested induction of systemic and local protective-memory immunity in concurrently *Mtb*-exposed HHCs.

The current study assessed the *Mtb* growth–controlling capacity of AMs and of autologous blood CD4 and CD8 T cells from HHCs. Autologous CD8 T cells from HHCs significantly increased the capacity of *in vitro*–infected AMs to control the growth of *Mtb*. AM, AM/CD4, and AM/CD8 cocultures produced IFN-γ and NO after infection with *Mtb*; there was, however, no correlation between IFN-γ and NO concentrations and the growth control of *Mtb* conferred by AM and CD8 T cells. Some of the results of these studies have been previously reported in the form of abstracts (52–54).

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METHODS

Study Subjects

HHCs of patients with active TB and CCs were screened for participation at the National Institute for Respiratory Diseases in Mexico City. Individuals willing to undergo bronchoalveolar lavage (BAL), venipuncture, HIV-1 serology, and TST were included in the study. A group of 13 HIV-1 seronegative HHCs (5 male, 8 female) were selected from first-grade relatives of acid-fast bacilli–positive patients with drug-sensitive TB. Each HHC shared the same house or room with an index patient for at least 3 mo before the patient's TB diagnosis. A group of 11 HIV-1 seronegative CCs without any known prior contact with patients with TB who had not been involved in patient care were recruited for the study. HHCs and CCs were 18 to 60 yr old. None of them had clinical or radiographic evidence of active pulmonary TB or of any other respiratory disease, or of acute, chronic, or immunocompromising diseases or therapies. Of the HHCs, eight were TST positive ($>$ 10 mm), as were three CCs; the remaining HHCs and CCs were TST negative ($<$ 10 mm).

Approval to obtain BACs by BAL and to perform venipunctures was given by the institutional review boards at the National Institute for Respiratory Diseases in Mexico City and the University of Medicine and Dentistry of New Jersey. Written, informed consent was obtained according to the guidelines of the U.S. Department of Health and Human Services.

Preparation of BACs

BACs were obtained by BAL as described previously (51). Briefly, after local anesthesia of the upper airways with 2% lidocaine solution and additional instillation of 1% lidocaine to prevent coughing, a flexible fiberoptic bronchoscope (P30; Olympus BF, New Hyde Park, NY) was wedged into the right middle lobe or the lingula. A total of 150 ml sterile 0.9% saline fluid was instilled in 20-ml aliquots into each of two subsegments. Mean yield of BAL fluid (BALF) was 78%. BALF was centrifuged at 400 $\times$ *g* for 15 min at 4°C. Pelleted BACs were resuspended in Roswell Park Memorial Institute (RPMI) 1640 (BioWhittaker, Walkersville, MD) with 50 μg/ml gentamicin sulfate, 200 mM L-glutamine, and 10% heat-inactivated pooled human serum (complete medium).

AMs were enriched from BACs using sheep red blood cells (SRBCs) (55) that bind to pan T-cell marker CD2. Briefly, 500 μl of washed SRBCs were activated with 1 IU neuraminidase (Sigma Chemical Co., Saint Louis, MO), and resuspended in RPMI. Then, 2 ml activated packed SRBCs and 2 ml fetal calf serum were added per milliliter of BACs (with 1–2 $\times$ 10^7 cells), and cell mixtures were incubated for 10 min at 37°C. Cells were then sedimented by centrifugation, incubated for 1 h on ice, and subsequently resuspended and overlaid on 3-ml Ficoll-Paque. After centrifugation, AMs were isolated from the buffy coat and washed three times. These cells were relatively lymphocyte-depleted and used as the AM population. Alveolar lymphocytes (ALs) were recovered from the pellet after lysis of the SRBCs with a buffer (0.01 M sodium bicarbonate–0.1 M ammonium chloride–0.1 mM ethylenediaminetetraacetic acid). The viability of BACs, AMs, and ALs was determined by exclusion of Trypan blue uptake, and was greater than 95% in both groups of subjects.

Preparation of PBMCs

Autologous PBMCs were prepared by density-gradient centrifugation of whole heparinized venous blood diluted 1:1 with RPMI over Ficoll-Paque (56) and resuspended in complete medium. The viability of PBMC was greater than 98% in each experiment from both groups of subjects.

Isolation of CD4 and CD8 Blood T Cells

Blood CD4 and CD8 T-cell subsets were prepared from PBMCs by stepwise immunomagnetic bead separation according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The mean purity of positively selected CD4 and CD8 T-cell preparations was 92 and 98%, respectively, as determined by fluorescence-activated cell sorting (FACS) analysis.

Preparation of $H_{37}Ra$ and $H_{37}Rv$ for *In Vitro* Infection

Suspensions of *Mtb* laboratory strains $H_{37}Ra$ and $H_{37}Rv$ (ATCC, Manassas, VA) were prepared in Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI) (57). After a 21-d incubation at 37°C, mycobacterial stock solution was harvested. To prepare a suspension with single bacteria and disrupt mycobacterial clump formation, bacterial pellets were then resuspended in RPMI with 6% glycerol and vortexed for 5 min in the presence of five sterile 3-ml glass beads. The resulting suspension of mycobacteria was centrifuged at 900 $\times$ *g* for 10 min to remove any remaining large clumps. Supernatants with disaggregated mycobacterial stock cultures were then divided into aliquots and stored at −70°C until use. Mean concentrations of the frozen $H_{37}Ra$ and $H_{37}Rv$ stock suspensions were determined by counting colony-forming units (cfu) on 7H10 agar plates in triplicate serial dilutions of declumped stock suspensions between Days 21 and 28. This declumping procedure was performed in each experiment to ensure use of single-bacterial-cell suspensions and to establish the input amounts of bacteria for the infections at the various multiplicities of infection (MOI).

In Vitro Infection and Intracellular Growth of $H_{37}Ra$ and $H_{37}Rv$ in AMs

AMs were plated in triplicate wells at a concentration of 10^5 cells/well in 96-well round-bottom microtiter plates (Corning, Inc., Corning, NY) and were adhered and rested in RPMI with 10% pooled human serum (PHS) overnight at 37°C in 5% CO_2. Cell-culture supernatants were carefully removed, and AMs were infected with $H_{37}Ra$ and $H_{37}Rv$ at MOI of 0.1 and 1, respectively. After a 1-h infection at 37°C in 5% CO_2, culture supernatants were removed, avoiding dislodging of the cells. Plates were then washed three times to remove any extracellular mycobacteria. Complete medium (200 μl) was then added to the wells for further culturing of the infected AMs. Cocultures of AMs (10^5 AMs/well) with autologous blood CD4 or CD8 T cells (AM/CD4 and AM/CD8 cocultures) were prepared by adding purified CD4 or CD8 T cells at ratios of 1:1 to both uninfected and infected AMs. Cell-culture supernatants were removed on Days 0, 4, and 7 for subsequent determination of IFN-γ, NO, and TNF-α by ELISA. AMs and AM/CD8 and AM/CD4 cocultures were then lysed with 0.1% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, MO) followed by addition of 7H9 and 20% bovine serum albumin (Sigma) on Days 0, 4, and 7. Serial dilutions of the lysates from triplicate wells from AMs and AM cocultures were then plated in triplicate onto Middlebrook 7H10 agar (Difco) and cfu were determined after a 21-d incubation at 37°C and 5% CO_2. In representative experiments, viability of cell cultures was assessed on Days 0, 4, and 7 by exclusion of Trypan blue uptake.

Average viabilities of AMs and AM cocultures in four representative experiments from both groups of subjects were as follows: Day 0, 95%; Day 4, 95%; and Day 7, 93%.

Phagocytosis of *Mtb* by AMs was assessed in additional wells by identification of acid-fast bacilli with Ziehl-Neelsen staining of AMs after 1 h of infection at 37°C in 5% CO_2 with $H_{37}Ra$ and $H_{37}Rv$ at MOI of 0.1 and 1. $H_{37}Ra$ and $H_{37}Rv$ phagocytosis at MOI 1 was similar (20–25%) for HHCs and CCs. At MOI 0.1, phagocytosis in HHCs was 11 to 15%, and in CCs it was 4%. Assessment of phagocytosis by cfu count on Day 0, however, revealed larger cfu numbers from HHC AMs than from CC AMs. The proportion of infected AMs was similar for HHCs and CCs; however, more bacteria had been phagocytosed by AMs from HHCs than by AMs from CCs.

NO Production

Culture supernatants from AMs and AM/CD4 and AM/CD8 cocultures were collected from 96-well plates at 1 h and on Days 4 and 7, divided into aliquots, and stored at −20°C until analysis. NO was quantified with the Griess reaction (58). Briefly, 50 μl of the culture supernatants were plated in 96-well flat-bottom plates and mixed with 50 μl of Griess reagent (0.1% naphtyl-ethilenediamine dihydrochloride and 1% sulfanilamide in 1 N HCl). The optical density was determined at 550 nm with a plate microreader (Labsystems Multiskan MCC/340; Labsystems, Helsinki, Finland). $NaNO_2$ was used to generate a standard curve. NO_2 concentrations in samples were expressed in μM/5 $\times$ 10^5 cells after background values with RPMI were subtracted.

IFN-γ and TNF-α ELISA

Culture supernatants from AMs and AM/CD4 and AM/CD8 cocultures from Days 0, 4, and 7 were assayed for IFN-γ and TNF-α. ELISA assays were performed using a final concentration of 1 μg/ml of antihuman IFN-γ (Endogen, Woburn, MA) or antihuman TNF-α capture antibody (Pharmingen, San Diego, CA). Ninety-six-well plates were then blocked with 100 μl Superblock (Pierce, Rockford, IL) at room temperature (RT) for IFN-γ and at 37°C for TNF-α. Standard curves with recombinant human IFN-γ (Endogen; 0–1,000 pg/ml) or TNF-α (Endogen; 0–2,000 pg/ml) were made, and 50 μl of standard dilution or cell-culture supernatant was added per well followed by incubation for 2 h at 37°C. After washing, biotinylated secondary anti–IFN-γ antibody (Endogen; 0.25 μg/ml) or anti–TNF-α antibody (Pharmingen; 0.5 μg/ml) was added and plates were incubated at RT for 1 h for IFN-γ and 45 min for TNF-α. Plates were washed and streptavidin-phosphatase conjugate (Jackson Immunoresearch, West Grove, PA) was added to each well. Plates were incubated 30 min at RT in the dark and developed with phosphatase substrate (Sigma) for 20 min at RT in the dark. The reaction was stopped with 5% ethylenediaminetetraacetic acid. Absorbance was read on a microplate reader (Labsystems Multiskan MCC/340; Labsystems) at 450 nm. ELISA results are presented as mean pg/ml from triplicate wells.

Analysis of Cell Surface Markers by FACS

The subpopulations of BACs and the purity of enriched AMs from BACs and of blood CD4 and CD8 T cells were assessed by FACS analysis using fluorescein isothiocyanate–labeled anti-CD3, CD4, CD8, CD56, γδ TCR, and CD14; phycoerythrin (PE)-labeled anti-CD69; and peridin-chlorophyll protein (PerCP)-labeled anti-CD3 antibodies. Fluorescein isothiocyanate–, PE-, and PerCP-labeled mouse anti-IgG1 and anti-IgG2a isotype antibodies were used to define quadrant positions in dot plots and percentage markers in histogram analyses. All antibodies were obtained from Becton Dickinson (San Jose, CA). Cells were antibody labeled, fixed with 1% paraformaldehyde, and kept at 4°C until acquisition in a FACScalibur (Becton Dickinson) within 24 h. Data analysis was done with the CELL-Quest software (Becton Dickinson). Cells were gated in the lymphocyte region (using FSC vs. SSC dot plot) to analyze the AL populations within the BACs. To assess the purity of AMs and of CD4 and CD8 blood T cells, no specific region (no gate) was selected.

Immunocytochemistry

Cellular profiles of BACs and AMs from HHCs and CCs on cytocentrifuge preparations were characterized using Wright's stain for nuclear morphology and a peroxidase stain that detects immature macrophages and neutrophils.

Statistical Analysis

Analyses to identify significant differences in cfu counts, IFN-γ, or NO concentrations between groups were done using a two-tailed Wilcoxon signed rank test, a nonparametric equivalent to a paired *t* test. Group means and SEM are presented. Statistical significance was set at $p < 0.05$, with all analyses being done using SPPS/PC+ version 11.5 (SPSS, Inc., Chicago, IL).

RESULTS

Subpopulations of BACs and Purity of AMs by Cytospin and FACS

By cytospin preparation, BACs from HHCs were 92% AMs and BACs from CCs were 90% AMs, with 8% ALs and less than 1% neutrophils; 5.5% of AMs from HHCs and 2% of AMs from CCs were peroxidase positive ($p = 0.041$). By FACS, ALs from HHCs were 47% $CD3^+CD4^+$, 22% $CD3^+CD8^+$, 9% $CD56^+$, and 3% $CD3^+$ $\gamma\delta TCR^+$. ALs from CCs were 42% $CD3^+CD4^+$, 29% $CD3^+CD8^+$, 10% $CD56^+$, and 5% $\gamma\delta TCR^+$.

Purity of AM populations after SRBC rosetting was 91% in HHCs and 92% in CCs by cytospin preparation. Blood T-cell populations from HHCs and CCs were 92.5 to 95% and 91 to 94% $CD3^+CD4^+$ and $CD3^+CD8^+$, respectively.

HHCs

Antimycobacterial activity of AMs and AM/CD8 cocultures. To test if concurrent aerogenic *Mtb* exposure of HHC induces bactericidal activity mediated by T cells on AMs, autologous blood CD8 and CD4 T cells from HHCs were added at a 1:1 ratio to *in vitro Mtb*-infected autologous AMs. Determinations of cfu from infections with $H_{37}Ra$ and $H_{37}Rv$ (MOI, 0.1 and 1) were made on Days 0, 4, and 7 in AMs and AM/CD8 and AM/CD4 cocultures.

Infection of AMs with $H_{37}Ra$ or $H_{37}Rv$ (MOI, 0.1) resulted in a steady cfu increase starting between 10^4 and 10^5 from Day 0 to Day 7 (Figures 1A and 1C). Interestingly, addition of CD8 T cells to AMs significantly increased control of $H_{37}Ra$ growth by AMs alone after 7 d. This observation was independent of the MOI (Figures 1A and 1B). CD8-mediated control of $H_{37}Rv$ growth was significant at MOI 0.1 (Figure 1C) but not at MOI 1 (Figure 1D).

There were no significant differences in the capacity of CD8 T cells from TST-negative and TST-positive donors to control *Mtb* growth.

In summary, CD8 T cells appear to increase *Mtb* growth control, particularly at MOI 0.1 and in $H_{37}Ra$ infections. The virulence of the $H_{37}Rv$ strain may have had an impact on the growth inhibition conferred by AMs and CD8 T cells as $H_{37}Rv$ cfu levels from AMs and AM/CD8 cultures were more than twofold greater than those from $H_{37}Ra$-infected cells.

IFN-γ and NO production by AMs and AM/CD8 cocultures. The importance of IFN-γ as an inducer of antimycobacterial activity of macrophages has been well established in the murine model. The current work assessed the production of IFN-γ in supernatants of AMs and AM/CD8 cocultures that were infected with $H_{37}Ra$ and $H_{37}Rv$. AMs produced IFN-γ after *in vitro* infection with either *Mtb* strain. IFN-γ concentrations in $H_{37}Ra$ and $H_{37}Rv$ (MOI, 0.1) infection increased similarly from Day 0 to

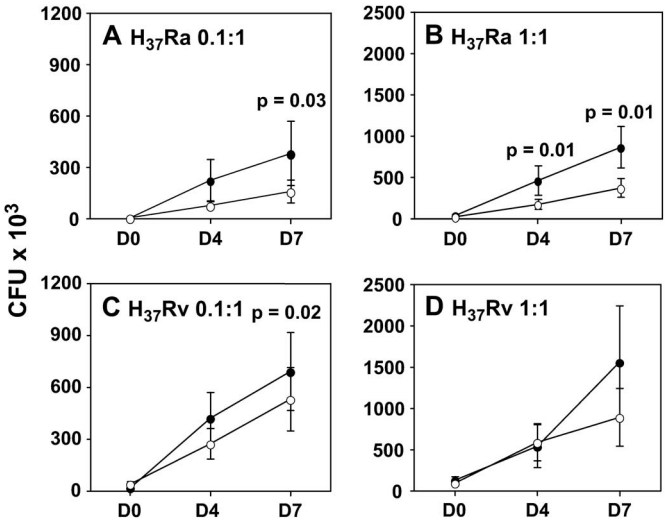

Figure 1. Inhibition by peripheral CD8 T cells of intracellular mycobacterial growth in alveolar macrophages (AMs) from healthy household contacts (HHCs) of patients with tuberculosis (TB). AMs alone (*closed circles*) and AMs in coculture with autologous CD8 T cells in a 1:1 ratio (AM/CD8; *open circles*) were infected with *Mtb* $H_{37}Ra$ ($n = 11$) or $H_{37}Rv$ ($n = 13$) at 0.1:1 (*A, C*) or 1:1 (*B, D*) bacteria/AM ratios for 1 h, 4 and 7 d (Days 0, 4, and 7). AM/CD8 colony-forming units (cfu) were lower than AM cfu in 8 of 11 subjects for $H_{37}Ra$ 0.1:1 (Day 7), and in 9 of 11 subjects for $H_{37}Ra$ 1:1 (Days 4 and 7). Mean cfu ± SEM cfu and significant differences ($p < 0.05$) are shown.

Day 7 (Figures 2A and 2C). AM infections with $H_{37}Rv$ and $H_{37}Ra$ at MOI 1 (Figures 2D and 2B, respectively) resulted in up to 30-fold increases from Day 0 to Day 7 ($p < 0.05$). IFN-γ levels of AMs were comparable at both MOI with $H_{37}Ra$ (Figures 2A and 2B).

IFN-γ concentrations in culture supernatants were affected by coculture of AMs with CD8 T cells, by the virulence of the infecting *Mtb* strain, and by the MOI.

IFN-γ concentrations in supernatants of infected AM/CD8 cocultures were one- to fourfold higher than that in AMs alone ($p < 0.05$).

IFN-γ levels in $H_{37}Rv$-infected AMs and AM/CD8 cocultures were between two- and threefold higher than those in $H_{37}Ra$-infected AMs and AM/CD8 cocultures (Figures 2A–2D). Regarding MOI, IFN-γ production was three- and twofold ($p < 0.05$) higher, respectively, in $H_{37}Ra$ and $H_{37}Rv$ infections with MOI 1 compared with infections with MOI 0.1 on Days 4 and 7. Constitutive IFN-γ levels in uninfected culture supernatants of AMs and AM/CD8 cocultures were low (means < 20 pg/ml) on Days 0, 4, and 7.

An important role for NO in the killing of *Mtb* and the significance of IFN-γ in the induction of NO production have clearly been established in the murine model. To assess in HHCs whether a relation existed between growth control of *Mtb* and NO, NO levels were measured in culture supernatants from AMs and AM/CD8 cocultures. AMs infected with $H_{37}Ra$ and $H_{37}Rv$ at MOI 0.1 produced low levels of NO (< 200 μM/10^5 cells); however, NO production was twofold higher in $H_{37}Rv$- compared with $H_{37}Ra$-infected AM cultures. NO levels increased from Day 0 to Day 7 in both strains at MOI 1 relative to MOI 0.1. NO production in AM/CD8 cocultures was lower than that in AM cultures alone (data not shown).

TNF-α production in AMs and AM/CD8 cocultures. TNF-α levels were assessed in supernatants from AMs alone and from AM/CD8 cocultures. Median TNF-α levels were twofold higher in AM/CD8 compared with AM cultures, indicating that CD8 T cells contribute to the production of TNF-α. Levels of TNF-α in AM/CD8 cultures were significantly higher in HHCs than in CCs ($p < 0.05$, data not shown). This observation suggests a role for TNF-α–producing CD8 effector cells in the growth control of *Mtb* in this model.

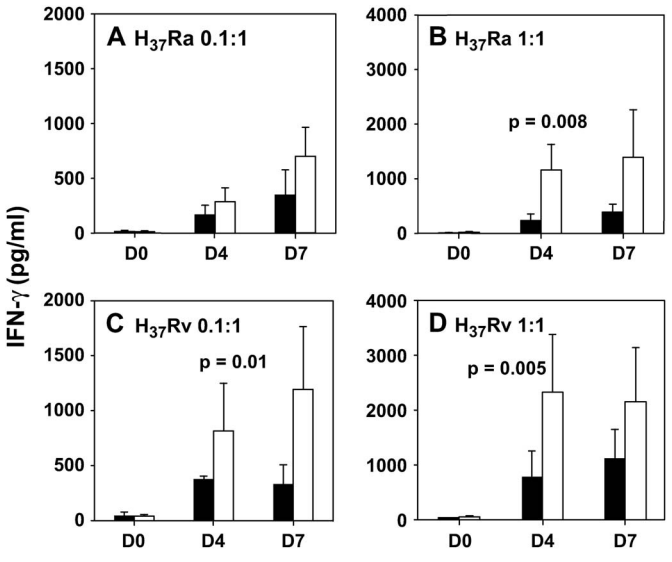

Figure 2. IFN-γ production in AMs and AM/CD8 cocultures from HHCs. IFN-γ production by AMs alone (*black bars*) or AMs in coculture with autologous CD8 T cells in a 1:1 ratio (AM/CD8; *white bars*) from HHCs was assessed in culture supernatants by ELISA on Days 0, 4, and 7 (Day 0, 4, and 7) (*A–D*). Experiments were performed as indicated in Figure 1. *Bars* represent mean values ± SEM. Significant differences between IFN-γ concentrations from AMs and AM/CD8 cocultures ($p < 0.05$) are shown.

Antimycobacterial activity and IFN-γ production in AMs and AM/CD4 cocultures. In a subgroup of HHCs (n = 4), growth control of $H_{37}Ra$ and $H_{37}Rv$ was studied in AM/CD4 cocultures (Figure 3). The intracellular growth of $H_{37}Rv$ and $H_{37}Ra$ at MOI 0.1 in AMs and AM/CD4 cocultures is shown. There was no significant difference between cfu in AMs and AM/CD4 cocultures (Figures 3A and 3B) with either $H_{37}Ra$ or $H_{37}Rv$, indicating lack of direct CD4 T-cell effects on *Mtb* growth in this model.

IFN-γ levels in AM/CD4 cocultures were higher compared with AM cultures both on infection with $H_{37}Ra$ and with $H_{37}Rv$, and increased from Days 4 to 7 (Day 4, $H_{37}Ra$, $p < 0.05$; Figures 3C and 3D). IFN-γ levels were also higher in AM/CD4 cocultures than in AM/CD8 cocultures, regardless of the infecting strain at MOI of 0.1 (not significant). Thus, as demonstrated by the lack of *Mtb* growth control conferred by CD4 T cells, increased IFN-γ production in AM/CD4 cocultures did not translate into improved growth control in AM/CD4 cocultures compared with AMs.

Healthy CCs

Antimycobacterial activity of AMs and AM/CD8 cocultures. In a different set of experiments, contribution of peripheral CD4 and CD8 T cells to the growth control of $H_{37}Ra$ and $H_{37}Rv$ in AMs from CCs was studied. Infection of AMs and AM/CD4 and AM/CD8 cocultures with $H_{37}Ra$ (Figure 4A) and with $H_{37}Rv$ (Figure 4B) at MOI of 0.1 showed minimal contribution of CD4 and CD8 T cells to the growth control conferred by AMs alone. There were no significant differences in cfu among AMs and AM/CD4 and AM/CD8 cocultures. Similarly, no CD4 or CD8 T-cell effect on *Mtb* growth control in AMs was seen with either of the strains at MOI 1 (data not shown).

Concentrations of IFN-γ in AMs and AM/CD4 and AM/CD8 cocultures. The production of IFN-γ in supernatants of AMs and AM/CD4 and AM/CD8 cocultures from CCs was also studied. Infection with $H_{37}Ra$ or $H_{37}Rv$ at MOI 0.1 (Figures 5A–5B) and MOI 1 (data not shown) induced similar increases of IFN-γ production by AMs from Day 0 to Day 7. As in HHCs, IFN-γ levels from AM/CD4 cocultures were significantly higher ($p < 0.05$) than those of AMs and AM/CD8 cocultures on Day 7 for all conditions: $H_{37}Ra$ and $H_{37}Rv$ at MOI 0.1 and MOI 1.

IFN-γ levels were higher in MOI 1 than in MOI 0.1 infections, regardless of the virulence of the strain (data not shown). Levels of constitutive IFN-γ production in uninfected AMs were low (< 50 pg/ml).

DISCUSSION

Healthy persons who are exposed to aerosolized *Mtb* allow for the study of early protective immunity in humans. To our knowledge, this study is the first of its kind to evaluate in HHCs the *in vitro* growth control of *Mtb* by *ex vivo* lung and blood cells. Growth control of avirulent $H_{37}Ra$ and virulent $H_{37}Rv$ by AMs alone and by AMs in coculture with autologous blood CD8 or CD4 T cells was assessed at the biologically relevant, low MOI of 0.1 and 1, which reduce mycobacterial clumping during infection and maximize AM viability *in vitro*.

Mtb growth in AM cultures from HHCs displayed a slow, steady increase of cfu over time. Addition of CD8 T cells to AM cultures, however, decreased $H_{37}Ra$ and $H_{37}Rv$ growth significantly. The *Mtb* growth-inhibiting effect of CD8 T cells on infected AMs was significantly larger than that conferred on

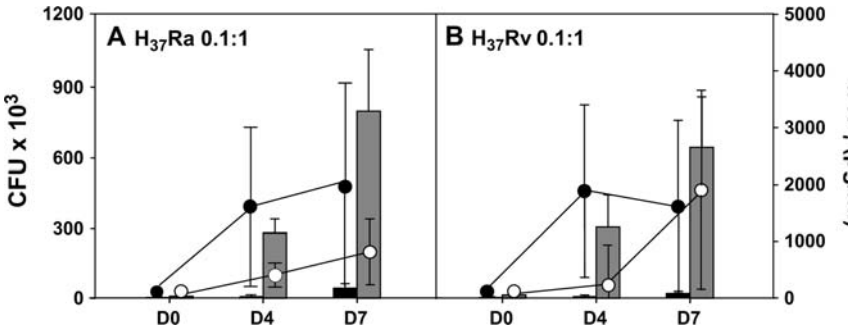

Figure 3. Effect of CD4 T cells on intracellular mycobacterial growth (cfu; *A*) and IFN-γ (*B*) production in AM cultures from HHCs (n = 4). AMs alone (*black circles*) or AMs in coculture with autologous CD4 T cells in a 1:1 ratio (AM/CD4; *white circles*) were infected with *Mtb* H_{37}Ra (*A*) or H_{37}Rv (*B*) at 0.1:1 bacteria/AM ratio for 1 h, 4 d, and 7 d (Days 0, 4, and 7), and cfu were determined. IFN-γ was assessed by ELISA in culture supernatants from AMs alone (*black bars*) and from AM/CD4 cocultures (*gray bars*). Mean cfu and IFN-γ concentrations ± SEM are shown.

AMs by CD4 T cells. Growth control was minimal when CD4 T cells were added to AM cultures in HHCs.

The greater *Mtb* growth control from CD8 compared with CD4 T cells in the current study contrasts with findings from Silver and colleagues (59). These investigators showed that CD4 T cells, rather than CD8 T cells, are responsible for growth control of H_{37}Rv in human MNs. This discrepancy may be attributable to differences in the model systems with use of AMs in our study and of MNs in theirs. Also, differences in intensity, time points, duration of subjects' exposure to *Mtb* (subjects in this study were concurrently exposed to *Mtb*), and the capacity of AMs and MNs to phagocytose *Mtb* (57) may explain the observed dissimilar results.

Because the importance of CD8 T cells in protection against *Mtb* infection has been clearly demonstrated in murine models of infection, the present study builds on observations (31–33) that *Mtb*-specific CD8 T cells are expanded in the *Mtb*-infected human and that these cells play an important role in *Mtb* growth control in AMs.

Besides their cytotoxic activity against infected target cells such as AMs (11) or MNs (9, 10), CD8 T cells contribute to protective immunity by secreting cytokines such as IFN-γ and TNF-α (12, 36, 60). To assess the concomitants of growth control of *Mtb* in this model system, the concentrations of IFN-γ, NO, and TNF-α were measured in the supernatants of AM cultures and of AM/CD8 and AM/CD4 cocultures. Concentrations of IFN-γ in AM/CD8 cocultures were higher than in AMs alone but lower than in AM/CD4 cocultures. This finding corroborates earlier reports that showed that *Mtb* antigen-specific human CD4 T cells are the primary source of IFN-γ (34) and that CD8 T cells contribute, although less than CD4 cells, to the overall production of IFN-γ (34, 38). IFN-γ production detected in cultures from AMs alone may in part have been from contamination with ALs, which was similar between HHCs and CCs (8%).

IFN-γ levels in cocultures of AMs with CD4 and CD8 T cells in HHCs and CCs appeared to be unrelated to the growth control of *Mtb* when correlated with cfu. This mirrors earlier findings in BACs from patients with TB in whom alveolitis of activated T cells (61) increased levels of locally produced IFN-γ, and frequencies of *Mtb* antigen-specific IFN-γ–producing cells at the site of disease (55, 62, 63) accompanied unhindered progressive immunopathology.

NO was detectable in AM supernatants from HHCs on Days 4 and 7, corresponding with earlier reports of NO production by AMs (46, 47), or in lung granulomas of patients with TB (49, 64) or in healthy individuals (48, 65). However, as with IFN-γ, there was no evidence for a positive correlation between NO concentrations and growth control of H_{37}Ra or H_{37}Rv in this study. NO concentrations in this study were slightly higher in H_{37}Ra than in H_{37}Rv culture supernatants but unaffected by the MOI of the infection. Thus, although IFN-γ is believed to be an inducer of NO, the present study did not show a direct positive correlation between IFN-γ and NO concentrations in culture supernatants.

Interestingly, TNF-α levels in culture supernatants were higher in AM/CD8 compared with AM cultures alone and higher in AM/CD8 cocultures from HHCs than AM/CD8 cocultures from CCs. These data suggest that TNF-α from CD8 effector T cells (66) may contribute to the observed limiting effect of CD8 T cells on *Mtb* growth.

The present study also showed that the capacity of *ex vivo* AMs and CD8 T cells to control *Mtb* growth was affected by the virulence of the infecting *Mtb* strain. Levels of cfu from H_{37}Ra-infected AMs were lower than those of H_{37}Rv-infected AMs on Days 4 and 7 when compared under the same experimental conditions. These findings coincide with earlier observations (67) of increased cfu levels in human MN-derived macrophages that were infected with H_{37}Rv or clinical isolates when compared with those infected with H_{37}Ra.

CD8 T cells play an important role in human antimycobacterial immunity. Despite *Mtb*-induced CD4 responses (IFN-γ production), only CD8 T cells enhance mycobactericidal capacity

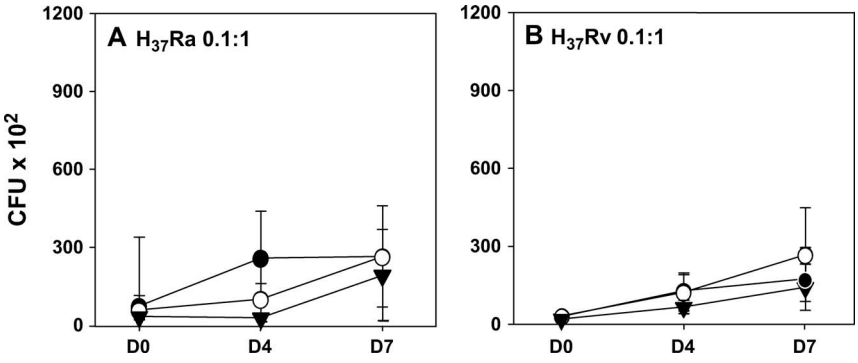

Figure 4. Effect of autologous peripheral CD8 and CD4 T cells on the mycobacterial growth in AMs from concurrent non–*Mtb*-exposed community control subjects (CCs). AMs (*black circles*) were infected with *Mtb* H_{37}Ra (*A*; n = 8) or H_{37}Rv (*B*; n = 9) at a 1:1 bacteria/AM ratio, and incubated alone or with autologous peripheral CD8 (*white circles*) or CD4 (*inverted black triangles*) T cells for 1 h (Day 0), 4 d (Day 4), or 7 d (Day 7). Mean cfu ± SEM are shown. No significant differences were found at any time point or condition tested.

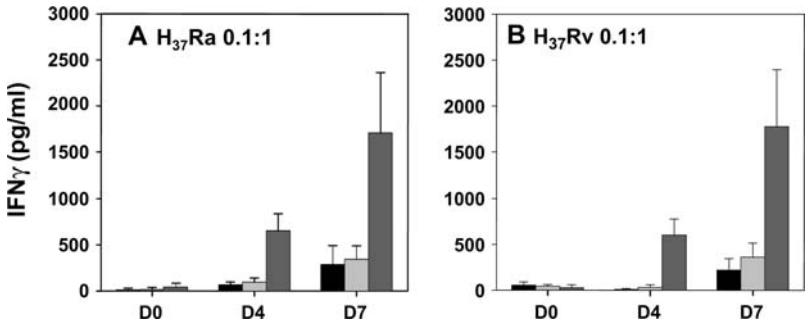

Figure 5. IFN-γ production by AMs alone (*black bars*) or by AMs in 1:1 coculture with autologous CD8 (AM/CD8; *light gray bars*) or CD4 (AM/CD4; *dark gray bars*) T cells in CCs. AMs were infected with *Mtb* H_{37}Ra (*A*; n = 8) or H_{37}Rv (*B*; n = 11) in the indicated conditions. Supernatants were harvested at the indicated time points and IFN-γ assessed by ELISA. *Bars* represent means ± SEM. Significant differences ($p < 0.05$) were found between AMs and AM/CD8 or AM/CD4 cocultures and between AM/CD8 and AM/CD4 cocultures.

of AMs in a uniform and significant manner in HHCs. The main reason for the improved capacity of CD8 T cells from HHCs to control *Mtb* growth may be the concurrent acute aerogenic exposure to *Mtb* and the subsequent expansion or activation of CD8 effector T cells. This notion is supported by a recent report of differential CD8 T-cell effector functions during acute and chronic phases of the immune response in the murine model. CD8 T cells isolated during the initial phase of infection showed lytic activity that is decreased in the chronic stage (68).

Recent evidence that human CD8 T cells preferentially recognize heavily infected cells (69) may suggest that differences in cfu levels of AMs from HHCs and CCs might have impinged on the CD8 T-cell effects on *Mtb* growth. However, differences in the model systems need to be considered. Lewinsohn studied infected dendritic cells that were exposed to CD8 T-cell clones and assessed IFN-γ release. In contrast, the current study assessed effects of *ex vivo* CD8-mediated control of *Mtb* growth in infected AMs that were not correlated directly with IFN-γ production, as IFN-γ production was similar in CCs and HHCs. Of interest also is the fact that CD8 T-cell activity controlling *Mtb* growth was lower in CCs than HHCs, although levels of cfu in AMs from CCs infected with MOI 1 (data not shown) were similar to those of AMs from HHCs infected with MOI 0.1. *In vivo* expansion and activation of CD8 T cells on *Mtb* exposure of HHCs thus contributed significantly to the observed differences between the study groups. However, during active *Mtb* infection, multiple factors including the degree of infection of antigen-presenting cells may determine expansion or activation of CD8 effector T cells.

In a set of separate studies from non–*Mtb*-exposed CCs, AMs were less permissive to *Mtb* infection because cfu levels were lower than those of AMs from HHCs. Differences in the activation status of AMs that resulted from concurrent *in vivo* exposure of HHCs to *Mtb* may be responsible for this finding. As in AMs from HHCs, *Mtb* cfu in AMs from CCs slowly increased between Day 0 and Day 4 with a plateau in growth between Day 4 and Day 7. However, no significant effect of CD8 or CD4 T cells was seen on this *Mtb* growth in AMs from CCs. These differences between HHCs and CCs were not due to variations in the input amount of bacteria or their preparation.

The limitations of this study are inherent in research on human subjects, particularly that involving the lung compartment. It is difficult to recruit large numbers of subjects and to control for extent and time point of exposure to *Mtb* in the household setting, which may result in variation of host immune responses among individuals. For small study groups, it may be explained that a statistically significant difference between TST-positive or TST-negative HHCs could not be detected. It is also important to consider that TST positivity in the study groups may have been due to both chronic *Mtb* infection or bacillus Calmette-Guérin vaccination (11 of 13 HHCs and 7 of 11 CCs had a bacillus Calmette-Guérin vaccination history) before the concurrent *Mtb* exposure period in the TB households. Processes leading to skin-test conversion may not translate directly into protection or improved effector T-cell activity. As suggested, the finding of CD8-mediated *Mtb* growth control that was observed here may be explained primarily by the recent *Mtb* exposure in the households. This exposure may have led to an expansion of *Mtb*-specific CD8 effector T cells, although additional mechanisms may have been at play. Longitudinal studies of larger cohorts of TB contacts may be required to assess immune responses in the lung in parallel with skin-test conversion and more specific *Mtb* antigen-based IFN-γ production assays.

In summary, together with earlier findings of increased protective-memory immunity in PBMCs (50) and BACs (51) of HHCs, this study shows that immune responses in HHCs are distinguishable from those of CCs. Furthermore, the presented *ex vivo* findings in HHCs suggest several processes that may occur in the intact host. First, *Mtb*-specific blood CD8 effector T cells may become expanded systemically after an exposure to *Mtb* or during ongoing aerogenic exposure. Second, these CD8 cells are capable of contributing to the control of *Mtb* growth in autologous *Mtb*-infected AMs. Third, recruitment and compartmentalization of the *Mtb*-specific CD8 T cells from the blood compartment to the local sites of inflammation in the lungs may play an important role in controlling the initial infection.

This work provides insights into human immunity that may require reinforcement with novel antituberculous vaccination or immunotherapeutic strategies. Antimycobacterial vaccination approaches that induce CD8 T-cell responses (70, 71) may be beneficial and particularly relevant in conditions of CD4-dependent immunodeficiencies.

***Conflict of Interest Statement*:** None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Niedergang F, Didierlaurent A, Kraehenbuhl JP, Sirard JC. Dendritic cells: the host Achille's heel for mucosal pathogens? *Trends Microbiol* 2004;12:79–88.
2. Sepkowitz KA. How contagious is tuberculosis? *Clin Infect Dis* 1996;23: 954–962.
3. Bates JH. Transmission and pathogenesis of tuberculosis. *Clin Chest Med* 1980;1:167–174.
4. Loudon RG, Bumgarner LR, Lacy J, Coffman GK. Aerial transmission of mycobacteria. *Am Rev Respir Dis* 1969;100:165–171.

5. Phillips L, Carlile J, Smith D. Epidemiology of a tuberculosis outbreak in a rural Missouri high school. *Pediatrics* 2004;113:e514–e519.
6. Comstock GW, Livesay VT, Woolpert SF. The prognosis of a positive tuberculin reaction in childhood and adolescence. *Am J Epidemiol* 1974;99:131–138.
7. Grzybowski S, Barnett GD, Styblo K. Contacts of cases of active pulmonary tuberculosis. *Bull Int Union Tuberc* 1975;50:90–106.
8. Shaw JB, Wynn-Williams N. Infectivity of pulmonary tuberculosis in relation to sputum status. *Am Rev Tuberc* 1954;69:724–732.
9. Canaday DH, Wilkinson RJ, Li Q, Harding CV, Silver RF, Boom WH. CD4(+) and CD8(+) T cells kill intracellular Mycobacterium tuberculosis by a perforin and Fas/Fas ligand-independent mechanism. *J Immunol* 2001;167:2734–2742.
10. Cho S, Mehra V, Thoma-Uszynski S, Stenger S, Serbina N, Mazzaccaro RJ, Flynn JL, Barnes PF, Southwood S, Celis E, *et al.* Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis. *Proc Natl Acad Sci USA* 2000;97:12210–12215.
11. Tan JS, Canaday DH, Boom WH, Balaji KN, Schwander SK, Rich EA. Human alveolar T lymphocyte responses to Mycobacterium tuberculosis antigens: role for CD4+ and CD8+ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. *J Immunol* 1997;159:290–297.
12. Brookes RH, Pathan AA, McShane H, Hensmann M, Price DA, Hill AV. CD8+ T cell-mediated suppression of intracellular Mycobacterium tuberculosis growth in activated human macrophages. *Eur J Immunol* 2003;33:3293–3302.
13. Rich EA, Panuska JR, Wallis RS, Wolf CB, Leonard ML, Ellner JJ. Dyscoordinate expression of tumor necrosis factor-alpha by human blood monocytes and alveolar macrophages. *Am Rev Respir Dis* 1989; 139:1010–1016.
14. Law K, Weiden M, Harkin T, Tchou-Wong K, Chi C, Rom WN. Increased release of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. *Am J Respir Crit Care Med* 1996;153:799–804.
15. Vankayalapati R, Wizel B, Weis SE, Samten B, Girard WM, Barnes PF. Production of interleukin-18 in human tuberculosis. *J Infect Dis* 2000;182:234–239.
16. Bonecini-Almeida MG, Ho JL, Boechat N, Huard RC, Chitale S, Doo H, Geng J, Rego L, Lazzarini LC, Kritski AL, *et al.* Down-modulation of lung immune responses by interleukin-10 and transforming growth factor beta (TGF-beta) and analysis of TGF-beta receptors I and II in active tuberculosis. *Infect Immun* 2004;72:2628–2634.
17. Saukkonen JJ, Bazydlo B, Thomas M, Strieter RM, Keane J, Kornfeld H. β-Chemokines are induced by Mycobacterium tuberculosis and inhibit its growth. *Infect Immun* 2002;70:1684–1693.
18. Kurashima K, Mukaida N, Fujimura M, Yasui M, Nakazumi Y, Matsuda T, Matsushima K. Elevated chemokine levels in bronchoalveolar lavage fluid of tuberculosis patients. *Am J Respir Crit Care Med* 1997;155: 1474–1477.
19. Sadek MI, Sada E, Toossi Z, Schwander SK, Rich EA. Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. *Am J Respir Cell Mol Biol* 1998;19:513–521.
20. Engele M, Stossel E, Castiglione K, Schwerdtner N, Wagner M, Bolcskei P, Rollinghoff M, Stenger S. Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent Mycobacterium tuberculosis. *J Immunol* 2002;168:1328–1337.
21. Boom WH, Canaday DH, Fulton SA, Gehring AJ, Rojas RE, Torres M. Human immunity to M. tuberculosis: T cell subsets and antigen processing. *Tuberculosis (Edinb)* 2003;83:98–106.
22. Daley CL, Small PM, Schecter GF, Schoolnik GK, McAdam RA, Jacobs WR Jr, Hopewell PC. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus: an analysis using restriction-fragment-length polymorphisms. *N Engl J Med* 1992;326:231–235.
23. Di Perri G, Cazzadori A, Vento S, Bonora S, Malena M, Bontempini L, Lanzafame M, Allegranzi B, Concia E. Comparative histopathological study of pulmonary tuberculosis in human immunodeficiency virus-infected and non-infected patients. *Tuber Lung Dis* 1996;77:244–249.
24. Lewinsohn DM, Bement TT, Xu J, Lynch DH, Grabstein KH, Reed SG, Alderson MR. Human purified protein derivative-specific CD4+ T cells use both CD95-dependent and CD95-independent cytolytic mechanisms. *J Immunol* 1998;160:2374–2379.
25. Serbina NV, Lazarevic V, Flynn JL. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection. *J Immunol* 2001;167:6991–7000.
26. Serbina NV, Flynn JL. CD8(+) T cells participate in the memory immune response to Mycobacterium tuberculosis. *Infect Immun* 2001;69:4320–4328.
27. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proc Natl Acad Sci USA* 1992;89:12013–12017.
28. Thoma-Uszynski S, Stenger S, Modlin RL. CTL-mediated killing of intracellular Mycobacterium tuberculosis is independent of target cell nuclear apoptosis. *J Immunol* 2000;165:5773–5779.
29. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A, Bogdan C, *et al.* An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 1998; 282:121–125.
30. Lazarevic V, Flynn J. CD8+ T cells in tuberculosis. *Am J Respir Crit Care Med* 2002;166:1116–1121.
31. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, Dockrell H, Pasvol G, Hill AV. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. *Proc Natl Acad Sci USA* 1998;95:270–275.
32. Mohagheghpour N, Gammon D, Kawamura LM, van Vollenhoven A, Benike CJ, Engleman EG. CTL response to Mycobacterium tuberculosis: identification of an immunogenic epitope in the 19-kDa lipoprotein. *J Immunol* 1998;161:2400–2406.
33. Lewinsohn DA, Lines RA, Lewinsohn DM. Human dendritic cells presenting adenovirally expressed antigen elicit Mycobacterium tuberculosis–specific CD8+ T cells. *Am J Respir Crit Care Med* 2002;166:843–848.
34. Canaday DH, Ziebold C, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation of human CD8+ alpha beta TCR+ cells by Mycobacterium tuberculosis via an alternate class I MHC antigen-processing pathway. *J Immunol* 1999;162:372–379.
35. Lewinsohn DM, Briden AL, Reed SG, Grabstein KH, Alderson MR. Mycobacterium tuberculosis-reactive CD8+ T lymphocytes: the relative contribution of classical versus nonclassical HLA restriction. *J Immunol* 2000;165:925–930.
36. Lewinsohn DM, Alderson MR, Briden AL, Riddell SR, Reed SG, Grabstein KH. Characterization of human CD8+ T cells reactive with Mycobacterium tuberculosis-infected antigen-presenting cells. *J Exp Med* 1998;187:1633–1640.
37. Heinzel AS, Grotzke JE, Lines RA, Lewinsohn DA, McNabb AL, Streblow DN, Braud VM, Grieser HJ, Belisle JT, Lewinsohn DM. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J Exp Med* 2002;196:1473–1481.
38. Shams H, Wizel B, Weis SE, Samten B, Barnes PF. Contribution of CD8(+) T cells to gamma interferon production in human tuberculosis. *Infect Immun* 2001;69:3497–3501.
39. Harty JT, Bevan MJ. Responses of CD8(+) T cells to intracellular bacteria. *Curr Opin Immunol* 1999;11:89–93.
40. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* 1993;178:2243–2247.
41. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* 1993;178:2249–2254.
42. Jouanguy E, Lamhamedi-Cherradi S, Altare F, Fondaneche MC, Tuerlinckx D, Blanche S, Emile JF, Gaillard JL, Schreiber R, Levin M, *et al.* Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. *J Clin Invest* 1997;100:2658–2664.
43. Jouanguy E, Altare F, Lamhamedi-Cherradi S, Casanova JL. Infections in IFNGR-1-deficient children. *J Interferon Cytokine Res* 1997;17:583–587.
44. Sharma S, Sharma M, Roy S, Kumar P, Bose M. Mycobacterium tuberculosis induces high production of nitric oxide in coordination with production of tumour necrosis factor-alpha in patients with fresh active tuberculosis but not in MDR tuberculosis. *Immunol Cell Biol* 2004;82: 377–382.
45. Bose M, Farnia P, Sharma S, Chattopadhya D, Saha K. Nitric oxide dependent killing of mycobacterium tuberculosis by human mononuclear phagocytes from patients with active tuberculosis. *Int J Immunopathol Pharmacol* 1999;12:69–79.
46. Kuo HP, Wang CH, Huang KS, Lin HC, Yu CT, Liu CY, Lu LC. Nitric oxide modulates interleukin-1beta and tumor necrosis factor-alpha synthesis by alveolar macrophages in pulmonary tuberculosis. *Am J Respir Crit Care Med* 2000;161:192–199.

47. Nicholson S, Bonecini-Almeida M, Lapa e Silva JR, Nathan C, Xie QW, Mumford R, Weidner JR, Calaycay J, Geng J, Boechat N, *et al.* Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med* 1996;183:2293–2302.
48. Rich EA, Torres M, Sada E, Finegan CK, Hamilton BD, Toossi Z. Mycobacterium tuberculosis (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. *Tuber Lung Dis* 1997;78:247–255.
49. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *Am J Respir Crit Care Med* 2002;166:178–186.
50. Torres M, Herrera T, Villareal H, Rich EA, Sada E. Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of Mycobacterium tuberculosis. *Infect Immun* 1998;66:176–180.
51. Schwander SK, Torres M, Carranza CC, Escobedo D, Tary-Lehmann M, Anderson P, Toossi Z, Ellner JJ, Rich EA, Sada E. Pulmonary mononuclear cell responses to antigens of Mycobacterium tuberculosis in healthy household contacts of patients with active tuberculosis and healthy controls from the community. *J Immunol* 2000;165:1479–1485.
52. Carranza C, Juarez E, Sarabia E, Torres M, Sada E, Ellner J, Schwander S. CD8 T cells from healthy household contacts of patients with active pulmonary tuberculosis (TB) improve growth control of M. tuberculosis H37Ra and H37Rv by autologous alveolar macrophages. Twelfth International Congress of Immunology and Fourth Annual Conference of FOCIS, Montreal, Quebec, July 18, 2004, W8.7.
53. Carranza C, Juarez E, Sarabia C, Escobedo D, Ellner J, Sada E, Schwander S. Growth control of *M.tuberculosis* by human alveolar macrophages and CD8 and CD4 lymphocytes from healthy household contacts of patients with active tuberculosis. U.S.–Japan Cooperative Medical Science Program, 38th Tuberculosis and Leprosy Research Conference, Newark, NJ, July 21–22, 2003.
54. Carranza C, Juarez E, Hernandez R, Sarabia C, Escobedo D, Ellner J, Sada E, Schwander S. Control by CD8 lymphocytes of growth of *M. tuberculosis* H37Ra in human alveolar macrophages from healthy household contacts of patients with active tuberculosis. Fourth World Congress on Tuberculosis, Washington, DC, June 3–5, 2002.
55. Schwander SK, Torres M, Sada E, Carranza C, Ramos E, Tary-Lehmann M, Wallis RS, Sierra J, Rich EA. Enhanced responses to Mycobacterium tuberculosis antigens by human alveolar lymphocytes during active pulmonary tuberculosis. *J Infect Dis* 1998;178:1434–1445.
56. Boyum A. Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol* 1976;Suppl 5:9–15.
57. Hirsch CS, Ellner JJ, Russell DG, Rich EA. Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of Mycobacterium tuberculosis by human alveolar macrophages. *J Immunol* 1994;152:743–753.
58. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982;126:131–138.
59. Silver RF, Li Q, Boom WH, Ellner JJ. Lymphocyte-dependent inhibition of growth of virulent Mycobacterium tuberculosis H37Rv within human monocytes: requirement for CD4+ T cells in purified protein derivative-positive, but not in purified protein derivative-negative subjects. *J Immunol* 1998;160:2408–2417.
60. Smith SM, Klein MR, Malin AS, Sillah J, Huygen K, Andersen P, McAdam KP, Dockrell HM. Human CD8(+) T cells specific for Mycobacterium tuberculosis secreted antigens in tuberculosis patients and healthy BCG-vaccinated controls in the Gambia. *Infect Immun* 2000;68:7144–7148.
61. Schwander SK, Sada E, Torres M, Escobedo D, Sierra JG, Alt S, Rich EA. T lymphocytic and immature macrophage alveolitis in active pulmonary tuberculosis. *J Infect Dis* 1996;173:1267–1272.
62. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. *Am J Respir Crit Care Med* 1998;157:729–735.
63. Robinson DS, Ying S, Taylor IK, Wangoo A, Mitchell DM, Kay AB, Hamid Q, Shaw RJ. Evidence for a Th1-like bronchoalveolar T-cell subset and predominance of interferon-gamma gene activation in pulmonary tuberculosis. *Am J Respir Crit Care Med* 1994;149:989–993.
64. Schon T, Elmberger G, Negesse Y, Pando RH, Sundqvist T, Britton S. Local production of nitric oxide in patients with tuberculosis. *Int J Tuberc Lung Dis* 2004;8:1134–1137.
65. Aston C, Rom WN, Talbot AT, Reibman J. Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide. *Am J Respir Crit Care Med* 1998;157:1943–1950.
66. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol* 2003;4:835–842.
67. Zhang M, Gong J, Lin Y, Barnes PF. Growth of virulent and avirulent Mycobacterium tuberculosis strains in human macrophages. *Infect Immun* 1998;66:794–799.
68. Lazarevic V, Nolt D, Flynn JL. Long-term control of Mycobacterium tuberculosis infection is mediated by dynamic immune responses. *J Immunol* 2005;175:1107–1117.
69. Lewinsohn DA, Heinzel AS, Gardner JM, Zhu L, Alderson MR, Lewinsohn DM. Mycobacterium tuberculosis-specific CD8+ T cells preferentially recognize heavily infected cells. *Am J Respir Crit Care Med* 2003;168:1346–1352.
70. Wang J, Santosuosso M, Ngai P, Zganiacz A, Xing Z. Activation of CD8 T cells by mycobacterial vaccination protects against pulmonary tuberculosis in the absence of CD4 T Cells. *J Immunol* 2004;173:4590–4597.
71. Lalvani A. CD8 cytotoxic T cells and the development of new tuberculosis vaccines. *Am J Respir Crit Care Med* 2002;166:789–790.